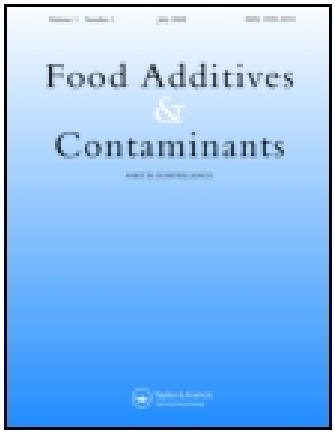

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Survey of benzoic acid in cheeses: contribution to the estimation of an admissible maximum limit

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Survey of benzoic acid in cheeses: contribution to the estimation of an admissible maximum limit

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Benzoic acid and its salts are commonly used additives in the food industry. Their use is not allowed in dairy products even though they can be found naturally. In this work, 100 cheese samples were tested to establish the maximum concentration that can be considered as ''natural'' and, therefore, permitted in cheeses. Analyses were carried out by a validated ion chromatography method and ''positive'' samples were confirmed by two other HPLC methods. Benzoic acid concentrations higher than the method LOQ ($8.8\,mg\,kg^{-1}$) were found in 18 samples, ranging from 11.3 to $28.7\,mg\,kg^{-1}$, with a mean value of $20.5\,mg\,kg^{-1}$. Taking into account the distribution of benzoic acid concentrations observed in ''positive'' samples, it is plausible to estimate a maximum admissible limit of $40.0\,mg\,kg^{-1}$ for benzoic acid in cheese. Below this value, samples can be considered ''compliant''.

Keywords: benzoic acid; benzoates; HPLC; ion chromatography; cheeses; survey; additives

Introduction

Benzoic acid and its salts are classified as food preservatives by European Parliament and Council Directive No. 95/2/EC (European Commission 1995). They exercise an antimicrobial activity by inhibiting some Krebs cycle enzymes, such as oxoglutarate deyhdrogenase and succinate dehydrogenase. They also inhibit some enzymes involved in oxidative phosphorylation (Chipley 1983).

These food preservatives can be added to several food products such as fish and meat products, sauces (mayonnaise, mustard), soft drinks, egg products, fruit-based food, vegetables, etc. European legislation has set specific limits for benzoic acid and its salts for each of these products. These limits range from $150\,mg\,l^{-1}$ for soft drinks to $5000\,mg\,kg^{-1}$ for egg products (European Commission 1995). These limits are high because these additives are considered not particularly harmful to humans. Several authors point out that benzoic acid at employment doses is harmless, does not cause accumulation problems and, in fact, is completely eliminated as hippuric acid by urine (Feldmann and Maibach 1970; US FDA 1972; Feillet and Leonard 1998). An acceptable daily intake (ADI) for benzoic acid and sodium benzoate of $0–5\,mg\,kg^{-1}$ body weight, corresponding to 300–400 mg for persons weighing 60–80 kg, has been established (FAO/WHO 1996). At these doses (300–400 mg for adults), benzoic acid is not toxic except for occasional allergic reactions to the active ingredient, such as rhinitis, hives and dermatitis, in sensitive persons (El-Ziney 2009). Excessive doses such as $1000\,mg\,day^{-1}$ for 5 consecutive days can cause nausea, headache and oesophagus burning.

Benzoic acid and benzoates are active as intermediary compounds in the synthesis of other substances (Goodwin 1976) and can be naturally present in many food products of vegetable and animal origin, such as dairy products (Sieber et al. 1989, 1990, 1995; European Commission 1995; Koyuncu and Uylaser 2009). This is because some fermented products contain benzoic acid coming from the fermentation process, even though the manufacturing practice is good (European Commission 1995). It could be derived from benzaldehyde that can be present at high concentrations in many cultured dairy products (Imhof et al. 1995). This substance is produced by certain strains of lactic acid bacteria or originates from phenylacetic acid oxidation by *Penicillium chrysogenum* (Hockenhull et al. 1952). Benzaldehyde is subject to auto-oxidation to benzoic acid in the presence of air (Bosset et al. 1982).

Nevertheless, benzoic acid and/or benzoate addition in dairy products (except acidulate milk, acidified milk, yogurt, kefir and buttermilk, for which a limit of $300\,mg\,kg^{-1}$ has been established), is not allowed by present European legislation. This can cause

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misinterpretation of inspection results regarding this product category. In fact, a sample could be considered as "non-compliant" even though it has never been treated with additives.

A research project has been proposed and developed to resolve such contradictions and fill this legislative gap. It was aimed at defining a maximum acceptable level of benzoic acid and benzoates in cheeses. Consequently, below this level, official laboratories should not have to communicate a "non-compliant" result. Similar studies have already been carried out for nitrates and nitrites in meat products (Bernini et al. 2001; Iammarino et al. 2005; Tanzi and Saccani 2005), for sulphites in fresh meat preparations (Iammarino et al. 2010) and for other substances, used as additives, but "naturally" present in food products.

Materials and methods

To correctly estimate such maximum acceptable level, benzoic acid was determined as benzoate ion by an high performance ion chromatography method, validated according to Regulation (EC) No. 882/2004 (European Commission 2004). A total of 100 cheese samples of different composition and origin were collected from several farms in the Apulia Region (Italy). To avoid collecting counterfeit cheeses, 88 samples were collected from farms that do not use food additives in their production technology. Good manufacturing practices were directly monitored by the authors. The remaining 12 samples were collected in local food stores, choosing D.O.P. products not containing food additives in their production process. Two replicates of each sample were analysed and the benzoic acid contents were evaluated as the mean of two measurements.

Chemicals and working standard solutions

Benzoic acid (99.9%) was supplied by Honeywell Riedel-de-Haën (Hanover, Germany). Carbonate-free sodium hydroxide (50%, w/w), sodium carbonate anhydrous (>99.5%) and methanol of HPLC-grade were purchased from JT Baker (Deventer, Netherlands). Acetic acid glacial (99.5%) was supplied by Scharlau (Barcelona, Spain). All solutions were prepared in ultrapure water with a specific resistance of 18.2 MΩ·cm, supplied by Milli-Q RG unit from Millipore (Bedford, MA, USA). Sodium hydroxide solutions used as eluents were prepared by dilution of a carbonate-free 50% (w/w) NaOH solution in water, previously filtered through a 0.45-μm membrane and degassed with nitrogen. From a benzoic acid standard solution at a concentration of 1000 mg l^{-1}, working standard solutions were prepared by dilution in ultrapure water to get the required concentrations.

Sample preparation

A 4-g portion of cheese sample, homogenised by a blade homogeniser, was mixed with 40 ml of ultrapure water and vortexed for 1 min. After centrifugation for 5 min at 1200 rpm at room temperature, the extract was filtered through Whatman No. 40 filter (Whatman, Springfield Mill, UK). About 2 ml were filtered through Anotop 10 LC, 0.2-μm, 10-mm filters (Whatman, Springfield Mill, UK) prior to chromatographic analysis. No further cleanup step was required.

Apparatus and method

Chromatographic separations were performed on a Dionex system (Dionex Corporation, Sunnyvale, CA, USA) composed of a GP50 quaternary gradient pump, an electrochemical detector (model ED40) set to conductivity mode equipped with a temperature compensated conductivity cell, and a Rheodyne injection valve (model RH9125, Cotati, CA, USA) with a 25-μl injection loop. A Dionex anion self-regenerating suppressor (ASRS II, 4 mm) was used for the electrochemical suppression at an operative current of 50 mA. Separations were performed using an IonPac AS11-HC column (250 × 4 mm I.D., particle size 13 μm; Dionex Corporation) eluted in gradient mode at a flow-rate of 1.0 ml min^{-1}. The mobile phase consisted of 38.25 mM NaOH (A) and 0.5 mM NaOH (B). The solvent program started with a linear gradient from 0 to 10% A in 12 min, isocratic for 5 min and quickly proceeded to 100% A in 1 min and remained for a further 3 min. Finally, the system was re-equilibrated for 3 min at 100% A. Plastic reservoir bottles (DX500 2-l bottles; Dionex) were closed and pressurised with pure nitrogen to 0.8 MPa. The system was interfaced via proprietary network chromatographic software (PeakNet™, Dionex Corporation) to a personal computer for instrumentation control, data acquisition and processing.

Validation procedures

The employed method for benzoic acid determination in cheeses has been validated by an in-house validation model, in agreement with Decision 657/2002/EC (European Commission 2002), with Regulation 882/2004/EC (European Commission 2004) and following Thompson harmonised validation guidelines (Thompson et al. 2002), which describe the analytical parameters to be appraised to assure method reliability. These parameters are selectivity, linearity, detection and quantification limits (LOD and LOQ), recovery, repeatability and ruggedness. The linearity test has been performed by three series of analyses on three different days, by injecting five benzoic acid standard solutions at concentrations of 1.0, 5.0, 10.0,

Table 1. Calibration parameters obtained by injections of standard solutions of benzoic acid.

$y = a + bx$				
$a \pm$ SD	$b \pm$ SD	r	LOD	LOQ
$(6.7 \pm 3.9)10^3$	$(321.1 \pm 3.1)10^2$	0.993	2.7	8.8

Notes: $y = a + bx$, where y is the peak area of benzoate ion and x is the concentration in mg l^{-1}.
r = correlation coefficient.
LOD and LOQ are expressed as benzoic acid in mg kg^{-1} of sample.

Table 2. Repeatability and recovery for the determination of benzoic acid in spiked cheese samples.

Fortification level[a]	Determined concentration (mean $\pm$ SD)	Recovery (%)	RSD_r (%)[b]	RSD_r reference (%)[c]
8.8 (LOQ)	8.4 ± 0.5	95.7	5.7	11.5
200.0	189.1 ± 7.2	94.6	3.8	4.8
300.0	291.8 ± 12.3	97.3	4.2	4.5
400.0	385.7 ± 16.3	96.4	4.2	4.3

Notes: [a]Expressed as mg kg^{-1} of benzoic acid. Six replicates at each fortification level.
[b]RSD_r: relative standard deviation under repeatability conditions.
[c]RSD_r reference was evaluated by the Horwitz equation as reported in Dec. 657/2002/EC.

30.0 and 60.0 mg l^{-1}. Linearity was verified in the range 1.0–60 mg l^{-1}, corresponding to 10–600 mg l^{-1} in the matrix, with correlation coefficients higher than 0.990. Calibration parameters are reported in Table 1, where standard deviations of slope and intercept are estimated at the 95% confidence level. Detection (LOD) and quantification (LOQ) limits were calculated according to the following equations (Miller 1993): $LOD = 3.3s_a/b$ and $LOQ = 10s_a/b$, where s_a is the standard deviation of the intercept and b is the slope of the regression line obtained from the calibration curve. LOD and LOQ values of 0.27 and 0.88 mg l^{-1}, respectively, were obtained. These values correspond to 2.7 and 8.8 mg kg^{-1} of benzoic acid in the matrix.

To test selectivity, 24 independent blank samples of cheeses (four hard cheeses, four semi-hard cheeses, four acid curd cheeses, four semi-soft cheeses, four soft cheeses and four pre-packed grated cheeses) have been analysed, to verify the absence of interfering peaks in the retention time-window of interest (±2.5% of benzoate ion retention time). Through these analyses, method selectivity toward matrix endogenous compounds has been demonstrated since, under the optimised separation conditions, a good separation of benzoic acid from endogenous compounds was found and the chromatogram is interference-free in the time-window where the benzoate ion elutes.

Repeatability and recovery have been determined by performing tests on three sets of blank semi-hard cheeses samples (six replicates each) fortified with benzoic acid at concentrations of 200, 300 and 400 mg kg^{-1} and at 8.8 mg kg^{-1} (corresponding to LOQ). The experiments have been performed on different days with the same instrument but different operators. In Table 2, repeatability values, calculated as result dispersion in terms of standard deviation (RSD_r) are given. By comparison with maximum standard deviations admitted by the Horwitz equation, as reported in Decision (EC) No. 657/2002, method repeatability was demonstrated. In Table 2, recovery data are also shown, calculated by comparing the concentration of spiked samples, determined by interpolation on the calibration curve with the nominal fortification level. Recovery values of 94.6, 97.3 and 93.4% at three fortification levels of 200, 300 and 400 mg kg^{-1}, respectively, were obtained with a mean value of 95.1%. In addition, repeatability and recovery values were also evaluated at low concentration level (8.8 mg kg^{-1} corresponding to LOQ). No significant difference was observed compared to data obtained at higher fortification levels (Table 2).

Method ruggedness under major changes conditions was evaluated by using Youden factorial experimental design (Youden and Steiner 1975). Therefore different sets of four hard cheese, four semi-hard cheese, four acid curd cheese, four semi-soft cheese, four soft cheese and four pre-packed grated cheese samples, spiked at a fortification level of 8.8 mg kg^{-1} (corresponding to LOQ), were prepared. The seven factors chosen as variables for the Youden test were the matrix and six fictitious factors; the use of a fictitious variable means no variation in analysis conditions. Therefore, the Youden experimental design requires 12 independent experiments: four with validation matrix (semi-hard cheeses) and four with each testing matrices. Analysis of hard cheeses gave a calculated standard deviation of difference S_{Di} of 1.1, lower than the estimated method precision ($S_{Di} = 2.4$), evaluated as twice the repeatability standard deviation of semi-hard cheeses samples at a fortification level of 8.8 mg kg^{-1}. The same consideration is valid for standard deviation values obtained for acid curd cheeses, semi-soft cheeses, soft cheeses and pre-packed grated cheeses ($S_{Di} = 0.8$, 1.1, 0.6 and 1.6, respectively). These results confirmed that the matrix variation has no effect on the analytical performances and, consequently, the method is also applicable to hard cheese, acid curd cheese, semi-soft cheese, soft cheese and pre-packed grated cheese samples. Figure 1 shows chromatograms of a hard cheese sample containing 11.8 mg kg^{-1} of benzoic acid (A) and a blank hard cheese sample (B).

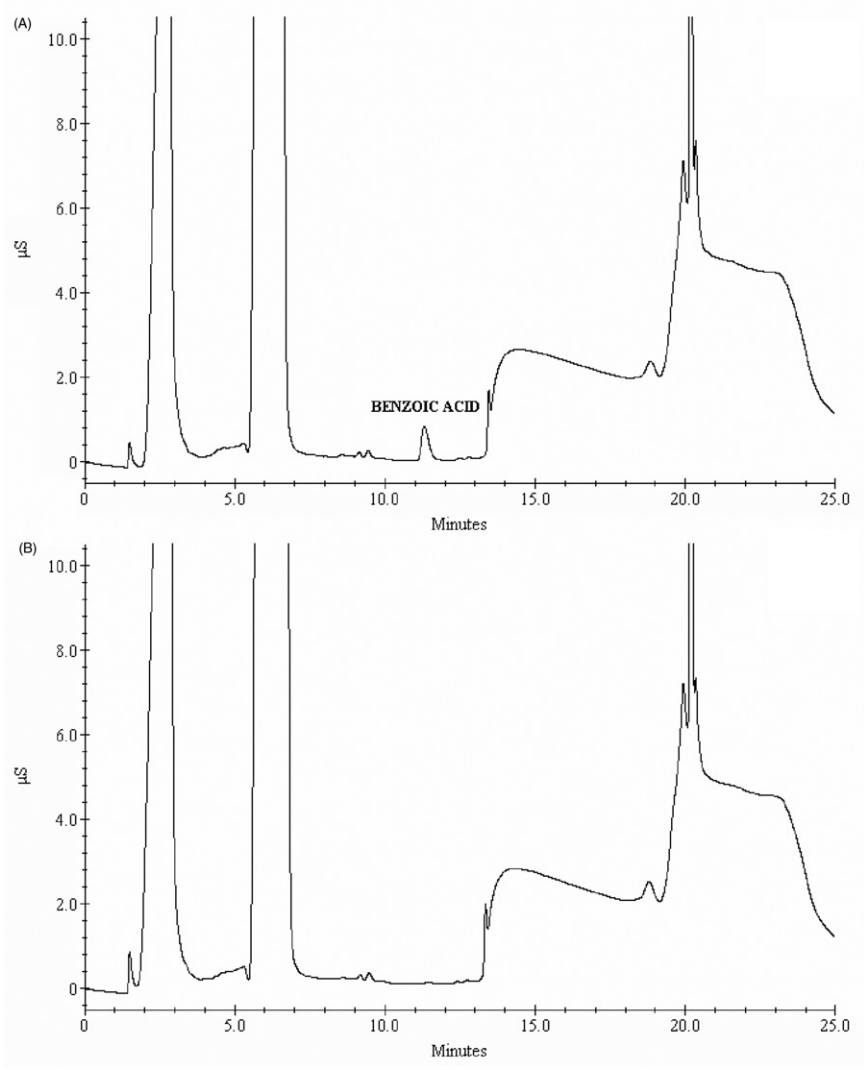

Figure 1. Chromatograms of a hard cheese sample containing 11.8 mg kg^{-1} of benzoic acid (A) and a blank hard cheese sample (B).

Evaluation of the uncertainty of the analytical results is compulsory for laboratories accredited according to ISO 17025 (ISO/IEC 2000) and several methods for the determination of this parameter have been proposed (EURACHEM/CITAC 2000). In this work, we have used the bottom-up method together with validation data obtained from each step of the analytical procedure (Hund et al. 2001). The concentration relative uncertainty has been calculated on the basis of uncertainties propagation law, by the equation:

$$\bar{u} = \sqrt{(\bar{u}(C))^2 + (\bar{u}(V_f))^2 + (\bar{u}(w))^2}$$

where u indicates the relative uncertainty, C is the analyte concentration in the sample, V_f is the volume of the final extract, and w is the sample weight. The determination of $u(C)$ has been performed by considering four sources of uncertainty: (a) preparation of the standards; (b) method repeatability; (c) method recovery; (d) calibration curve. By using a coverage factor k of 2, corresponding approximately to a 95% confidence level, a relative expanded measurement uncertainty of 8.2% was obtained, confirming the laboratory technical competence and the method reliability in the quantitative determination of benzoic acid in the cheeses. For repeatability and recovery uncertainties estimation, data obtained from 10 repeated analyses of a semi-hard cheese sample fortified with benzoic acid at a concentration of 8.8 mg kg^{-1} were used.

Confirmatory analyses using different chromatographic methods

To confirm the results obtained by validated method, "positive" samples were also analysed using two alternative chromatographic methods.

Ion chromatography separations by IonPac AS9-HC column (Dionex Corporation, Sunnyvale, CA, USA)

For this type of confirmation analyses, the same chromatographic system and the same extraction and purification procedures of the validated method were employed. The separations were performed by isocratic elution with 9 mM Na_2CO_3 at a flow-rate of 1.0 ml min^{-1} (total run time: 20 min). The anion self-regenerating suppressor (ASRS II, 4 mm) was used for the electrochemical suppression, at an operative current of 50 mA. Similar results were obtained and all "positive" samples were confirmed by this alternative ion-chromatography method.

Reversed-phase liquid chromatography separations

The second alternative method used for the confirmation analyses was based on reversed-phase liquid chromatography and UV detection. Chromatographic separations were performed on a HPLC system, Waters™ 2690 Separations Module (Milford, MA, USA) equipped with a Waters™ 996 PDA d, a micro vacuum degasser, an autosampler and a column compartment. All the separations were performed by using a Luna C_{18} column (250 × 4.6 mm I.D., particle size 5 μm; Phenomenex, Torrance, CA, USA) operating at a flow-rate of 1.5 ml min^{-1} under isocratic elution with a mixture of water, methanol and acetic acid (69:28:3, v/v); total run time: 40 min. The injection volume was 20 μl. UV detection was performed at 275 nm and the system was interfaced, via network chromatographic software (Millennium^{32}, Waters™ Milford, MA, USA) to a personal computer. All samples analysed "positive" by the validated method were also confirmed by this second confirmation method. Figure 2 shows chromatograms confirming the reported analysis of a benzoic acid-contaminated sample.

Results and discussion

A total of 100 samples of cheeses were analysed, including the most common brands (D.O.P. products, 12 samples) and products coming from local dairy farms (88 samples). They were made without preservatives under direct control of the authors and were characterised by various production technologies, raw materials and places of production. To simplify data processing, the cheeses were subdivided into six categories according to Burkhalter (Early 1998). The cheese categories were as follows: hard cheeses (17 samples), semi-hard cheeses (18 samples), soft cheeses (14 samples), semi-soft cheeses (24 samples), acid-curd cheeses (15 samples) and pre-packed grated cheeses (12 samples).

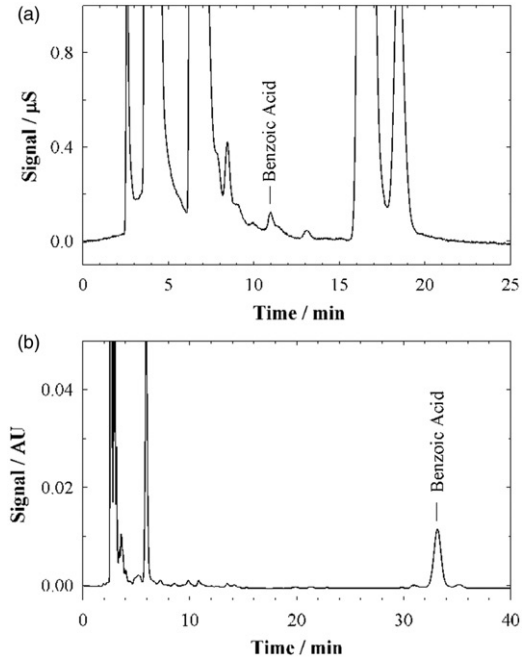

Figure 2. Confirmatory analyses of a "non-compliant" cheese sample. (a) Ion chromatography with weak ionic exchange and conductivity detection. (b) Reversed-phase liquid chromatography with UV detection at 275 nm.

The wide range of analysed samples allows us to draw valid conclusions for most types of cheese. The obtained data, subdivided by category, milk type and ripening time of analysed cheeses, expressed in terms of benzoic acid concentrations in "positive" samples and number of "positive" samples for each typology are reported in Table 3.

By data processing, benzoic acid presence was registered in each of six analysed categories and at least one "positive" sample was recorded for each category. Therefore, it is not possible to exclude, *a priori*, the "natural" presence of benzoic acid in any cheese typology, whether it is made from raw or pasteurised milk, rapid or lengthy maturation, produced with cow's, sheep's or goat's milk. Benzoic acid generation in cheese seems to derive from common factors to all cheese typologies and does not seem to be easily controllable, since benzoic acid can be present regardless of production technology and raw material characteristics.

Nevertheless, quantifiable amounts of benzoic acid were obtained in only 18 samples and these values were characterised by low levels (mean concentration was 20.5 mg kg^{-1}, maximum value was 28.7 mg kg^{-1}). Consequently, it is possible to conclude that there are no significant risks for humans in relationship to benzoic acid development in cheeses.

A legislative gap exists, taking into account the distribution and the entity of the observed concentrations, considering the measurement uncertainty of the

Table 3. Results from the analysis of 100 cheese samples.

Cheese category	Milk type	Ripening time (months)	Number of analysed samples	Number of "positive" samples	Benzoiz acid concentrations of "positive" samples[a]		
Hard cheeses (17 samples)	Cow/raw	16	2	0		–	
	Cow/raw	18	2	1	11.8 ± 1.0		
	Sheep/raw	16	2	0		–	
	Sheep/raw	18	7	1	11.3 ± 0.9		
	Sheep/raw	18	4	0		–	
Semi-hard cheeses (18 samples)	Cow/raw	8	7	2	25.7 ± 2.1	25.8 ± 2.1	
	Sheep/raw	8	2	0		–	
	Sheep/raw	12	3	1	20.3 ± 1.7		
	Cow/pasteurised	16	2	1	22.6 ± 1.9		
	Cow/pasteurised	8	2	0		–	
	Cow/pasteurised	8	2	0		–	
Soft cheeses (14 samples)	Cow/raw	1	8	2	19.8 ± 1.6	22.3 ± 1.8	
	Sheep/raw	1	3	0		–	
	Cow/pasteurised	1	3	1	24.5 ± 2.0		
Semi-soft cheeses (24 samples)	Cow/raw	6	3	0		–	
	Cow/raw	6	5	2	12.5 ± 1.0	28.7 ± 2.4	
	Cow/raw	2	4	0		–	
	Goat/raw	2	5	1	21.0 ± 1.7		
	Cow/pasteurised	2	5	1	16.3 ± 1.3		
	Cow/pasteurised	6	2	1	22.3 ± 1.8		
Acid-curd cheeses (15 samples)	Cow/pasteurised	2	5	0		–	
	Cow/pasteurised	3	3	0		–	
	Cow/pasteurised	6	7	1	22.2 ± 1.8		
Pre-packed cheeses (12 samples)	–	–	12	3	17.1 ± 1.4	24.0 ± 2.0	25.8 ± 2.1

Note: [a]Expressed as $mg\,kg^{-1}$ of benzoic acid $\pm$ expanded measurement uncertainty percentage.

method (8.2%) and adopting a reasonable tolerance it is possible to estimate for benzoic acid a maximum admissible limit of $40.0\,mg\,kg^{-1}$ in cheeses. As a result, the sample can be considered "compliant" when its benzoic acid concentration is below the proposed limit, because such a low concentration could originate from natural endogenous formation, due to particular biochemical mechanisms during cheese maturation and not from fraudulent addition.

Conclusions

A survey of 100 cheese samples of varying composition and origin focused on tracing quantifiable concentrations of benzoic acid was carried out by a validated ion chromatography method to establish a maximum allowable limit.

Although benzoic acid can be found in any typology of cheese, regardless of production technology, origin of milk and production area, quantifiable concentrations were obtained in only 18 samples at low levels (mean concentration: $20.5\,mg\,kg^{-1}$; maximum concentration: $28.7\,mg\,kg^{-1}$). So, it is possible to conclude that there are no significantly risks for humans in relationship to benzoic acid development in cheeses.

Taking into account the level of benzoic acid in "positive" samples, it is plausible to estimate a maximum admissible limit of $40.0\,mg\,kg^{-1}$ in cheeses. Below this value, the cheese sample can be considered "compliant."

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